EFFECT OF EXPOSURE TIME, BACTERIA CONCENTRATION AND CULTURE AGE OF *ESCHERICHIA COLI* AND *BACILLUS SUBTILIS* ON THE GLASS SURFACE

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ABSTRACT

This thesis presents the effect of the bacterial adhesion on the glass surface (hydrophilic surfaces) at different time exposure and bacterial concentration. The ability of *Escherichia* coli and *Bacillus subtilis* to attach to the surfaces depends mainly on the interaction of hydrophobic domains. However, *E. coli* and *B. subtilis* have evolved in different ways in order to manipulate the hydrophobic effect for their adherence on the solid surface. On the other hand, the surface properties e.g surface charges are inherently important and often regulate the mechanism of the bacteria adhesion. Besides that, adhesions of bacteria were also affected by culture media, exposure time of bacteria on glass surface, age and bacterial concentration. Both bacteria have different surface characteristic which also affect adhesion on the glass surface. Both bacteria were suspended in the phosphate buffer solution (pH 7.1) at different cell concentration (abs). The solution was suspended into glass container containing glass slide. The glass-bacterial solution was shake at 100 rpm and 30^0C in the incubator shaker and sampling were done at 4 h, 8 h, 12 h and 24 h. From the researches that have been done *B. subtilis* easily adhere on the glass surface compared to *E. coli*, with 46.9% reduction in optical density reading observed at 600nm. *Bacillus subtilis* was exposed for 24 hour at cell concentration 0.8 abs. Meanwhile, *E. coli* result in less adhesion to the glass surface with only 29.8 % reduction in optical density. Yet, the time of exposure for *E. coli* was only 12 hour with cell concentration 1.0 abs.

ABSTRAK

Tesis ini membentangkan kesan lekatan bakteria pada permukaan kaca (permukaan hidrofilik) pada pendedahan masa yang berbeza dan kepekatan bakteria yang berbeza. Keupayaan *Escherichia coli* dan *Bacillus subtilis* untuk melekat pada permukaan bergantung terutamanya kepada interaksi domain hidrofobik. Walau bagaimanapun , *E. coli* dan *B. subtilis* berinteraksi dengan cara yang berbeza untuk memanipulasi kesan hidrofobik untuk pelekatan mereka di permukaan pepejal. Sebaliknya, sifat-sifat permukaan seperti caj permukaan sememangnya penting dan sering mengawal mekanisme lekatan bakteria . Di samping itu, pelekatan bakteria turut terjejas olehfaktor sekeliling, masa dedahan bakteria pada permukaan kaca , umur dan kepekatan bakteria. Kedua-dua bakteria tersebut mempunyai ciri permukaan yang berbeza yang juga mempengaruhi lekatan pada permukaan kaca. Kedua-dua bakteria dimasukkan dalam penyelesaian penimbal fosfat (pH 7.1) pada kepekatan sel yang berbeza (abs). Bakteria yang dicampur dengan Phosphate buffer solution (PBS) telah dimasukkan ke dalam bekas kaca yang mengandungi kepingan kaca. Kepingan kaca-bakteria digoncang pada 100 rpm dan 30 ° C dalam penggoncang inkubator dan pemerhatian pelekatan bakteria pada kepingan kaca dilakukan pada jam ke-4 , ke-8, ke-12 dan ke-24 . Dari kajian yang telah dilakukan *B. subtilis* lebih mudah melekat pada permukaan kaca berbanding dengan *E. coli*, dengan pengurangan 46.9 % dalam membaca ketumpatan optik diperhatikan pada 600nm . *B. subtilis* telah didedahkan selama 24 jam di kepekatan sel 0.8 abs. Sementara itu , *E. coli* kurang lekatan ke permukaan kaca dengan pengurangan hanya 29.8 % dalam ketumpatan optik. Namun , masa pendedahan bagi *E. coli* adalah hanya 12 jam dengan kepekatan sel 1.0 abs.

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LIST OF ABBREVIATION

E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*
OD	Optical Density
Abs	Absorbance
PBS	Phosphate Buffer solution
T_0	Time at 0^{th} hour
T_4	Time at 4^{th} hour
T_8	Time at 8^{th} hour
T_{18}	Time at 18^{th} hour
T_{24}	Time at 24^{th} hour

1 INTRODUCTION

1.1 Motivation and problem statement

A fundamental question often asked ‘why do microorganisms stick to a surface?’ The prime directive of microorganism is to reproduce and to do so they must assimilate nutrient in sufficient amount to ensure that the process is successful. Almost all biological processes require an aqueous environment including the transport of nutrient into the microbial cell. Bacteria adhesion is the initial step of colonization and formation of biofilm. It causes an accumulated biomass of microorganism and extracellular material on certain area of the solid surfaces, where it depend on a number of microbiological, physical, chemical and material-related parameters. The ability to stick onto a surface would immediately provide several advantages to ensure reproduction in a nutrient limiting environment. Microbial adhesion is not limited to hard, intimate surfaces, but applicable even to soft tissues. For instance, human skin intestinal and pulmonary lining and urinary tract are all colonizable by microorganism which may result in pathologies

Over the past few decades, biofilm formation has been observed in many industrial and domestic domains. Unfortunately, in most cases the growth of biofilms has been detrimental, where many industries suffers the ill-effects of biofilm growth which result in heavy costs in cleaning and maintenance. Industries such as maritime, dairy (Yoo, 2002), food (Ganesh. 1998), water systems (Bott, 1998), oil (Nemati, 2001), paper (Klahre, 2000), opticians (Liesegang, 1997), dentistry (Marotta, 2002) and hospitals (Halabi, 2001) which often involved billions of dollars for cleaning and maintenance services . Perhaps the environment where people are exposed to biofilms most frequently is the domestic environment (Baker, 2000). Product spoilage, reduced production efficiency, corrosion, unpleasant odours (malodours), unsightliness, infection, pipe blockages and equipment failure are examples of the detrimental effects of biofilms. For these reasons and the emergence of restrictive legislation regarding the effects of cleaning agents on the environment and to user health and safety (Commission Regulation EC No. 1048/ 2005),

there is a lot of industrial interest in developing materials and methods which can remove and actively prevent the formation of biofilms.

In the UK, it is estimated that 9 million cases of intestinal disease every year, much of which originates at home, where human excreta are the primary source of infection (Curtis, 2003). Estimates show that for every case of infectious disease reported to the Communicable Disease Surveillance Centre (CDSC), 136 unreported cases occur in the community causing considerable morbidity. In the food industry biofilms cause serious engineering problems such as impeding the flow of heat across a surface, increases in fluid frictional resistance of surfaces and increases in the corrosion rate of surfaces leading to energy and production losses. Pathogenic microflora grown on food surfaces and in processing environments can cross-contaminate and cause post-processing contamination (Verran, 2000). If the microorganisms from food-contact surfaces are not completely removed, they can lead to mature biofilm formation and so increase the biotransfer potential. Examples of the food sectors that pay particular attention to the possibility of cross-contamination are the milk industry (Chye, 2004) and the slaughter industry.

Virulence and pathogenicity of microorganisms is often enhanced when growing as a biofilm, and new strategies are therefore required to control biofilm formation and development. Many pathogenic microorganisms reside within biofilms, which biofilms cause additional problems when designing new anti-microbial agents. Novel strategies are necessary because of the limitations to these current treatments such as inadequate control supply, potential for disease transfer and compliance issue. The capability and high resistance of sessile microorganisms to inhibitors, eradication of biofilm often requires high concentration of disinfectants or antibiotics, causing severe environmental damages, multi-resistance emergence and nosocomial infections. Public health concerns, as well the economic loss associated to biofilm formation raise an urgent need for developing biofilm resistant systems.

The adhesion of bacteria on the solid surfaces have causes a lot of problems. Indeed the adhesive characteristics of natural human flora are now considered as a tool for preventing the adhesion of pathogenic bacteria to avert infection. To eliminate this

problem, studies on developing the anti-adhesive surfaces, incorporation of anti bacteria agent into medical device polymer, mechanical design alternative and produce antibiotic had bloomed significantly (Geesey, 2001; von Eiff *et al.*, 2002; Vincent, 2003; Lejeune, 2003). The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables such as surface roughness, chemical stability, hydrophobicity and surface charge (Donlan, 2002). In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Martial, & Degraeve, 2008, Simo˜es, 2008). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and EPS production are important for biofilm formation and development (Parsek & Greenberg, 2005). An increase in flow velocity or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (Simo˜es, Sillankorva, et al., 2007).

1.2 Objective

In order to manipulate the occurrence of bacteria adhesion and biofilm formation, it is of important to study the factors that contribute to the bacteria adhesion on the solid surfaces. To study the factors that facilitates the adhesion of bacteria (*Escherichia coli* and *Bacillus subtilis*) on the glass surface (hydrophilic surfaces).

1.3 Scope

The scope have been drawn where bacteria characterization is characterized based on the types, morphology, size and shape. Besides that, the physical effects on bacteria adhesion; exposure time (4, 8, 12 and 24), bacterial concentration (0.8, 1.0 and 1.2) abs and culture age (16 and 66 hour).

2 LITERATURE REVIEW

2.1 Microorganism

2.1.1 Escherichia coli

Escherichia coli is a gram negative procaryote, non-spore forming rod. It may or may not be mobile. (Some rods are flagellated and some are not.) The organism is a facultative anaerobe and the optimal temperature for growth is at 37^{o}C. The optimum pH for growth is 6.0 to 8.0. However, growth can occur as low as pH 4.3 and as high as pH 9 to 10. *E. coli* is prokaryotic and capable of aerobic and anaerobic metabolism. *E. coli* is a heterotrophic organism, meaning that it obtains its food from a different source. This source is most often its host organism. They obtain carbon via biosynthesis of organic molecules that were ingested by their host. Carbon is very important to *E. coli* because the bacterial cell composed almost entirely of carbon molecules bound to other important elements. In response to changes in the temperature or the osmolarity of the environment, *E. coli* utilizes its ability to physically change the diameter of the porins found on the cell membrane. If there are larger nutrient molecules present, *E. coli* will enlarge in porin diameter of to allow the molecule to enter the organism. This also works in reverse in that if there are inhibitory molecules present, *E. coli* will decrease the diameter of the porins **(**Hu Amanda, 2002).

2.1.2 Bacillus subtilis

Bacillus subtilis cells are rod-shaped, gram-positive bacteria that are naturally found in soil and vegetation. *B. subtilis* grows best in the mesophilic temperature range where the optimal temperature is 25 to 35^{o}C (Stephen, 1998). Stress and starvation are common in this environment; therefore, *B. subtilis* has evolved a set of strategies that allow survival under these harsh conditions. For example, is the formation of stress-resistant endospores. Besides that, the other strategy is the uptake of external DNA, which allows the bacteria to adapt by recombination. However, these strategies are time-consuming. *B. subtilis* can also gain protection more quickly against many stress situations such as acidic, alkaline,

osmotic, or oxidative conditions, and heat or ethanol (Bandow, 2002). *B. subtilis* use their flagella for a swarming motility. This motility occurs on surfaces, for example on agar plates, rather than in liquids. *B. subtilis* are arranged in singles or chains. Cells arranged next to each other can only swarm together, not individually. These arrangements of cells are called 'rafts'. In order for *B. subtilis* to swarm, they need to secrete a slime layer which includes surfactin, a surface tension-reducing lipopeptide, as one of its components (Schaechter 2006).

2.2 Growth curve

Binary fission and other cell division processes bring about an increase in the number of cells in a population. Population growth is studied by analyzing the growth curve of a microbial culture. When microorganisms are cultivated in liquid medium, they usually are grown in a batch culture that is, they are incubated in a closed culture vessel with a single batch of medium. Because no fresh medium is provided during incubation, nutrient concentrations decline and concentrations of wastes increase. The growth of microorganisms reproducing by binary fission can be plotted as the logarithm of the number of viable cells versus the incubation time (Ingraham,2001).

2.2.1 Lag Phase

When microorganisms are introduced into fresh culture medium, usually no immediate increase in cell number occurs. This period is called the lag phase**.** However, cells in the culture are synthesizing new components. A lag phase can be necessary for a variety of reasons. The cells may be old and depleted of ATP, essential cofactors, and ribosome; these must be synthesized before growth can begin. The medium may be different from the one the microorganism was growing in previously. Here new enzymes would be needed to use different nutrients. Possibly the microorganisms have been injured and require time to recover. Whatever the causes, eventually the cells begin to replicate their DNA, increase in mass, and finally divide (Neidhardt, 2005).

2.2.2 Exponential phase

During the exponential (log) phase**,** microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions. Their rate of growth is constant during the exponential phase; that is, they are completing the cell cycle and doubling in number at regular intervals. The population is most uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical and physiological studies (Neidhart, 2005).

Exponential (logarithmic) growth is balanced growth**.** That is, all cellular constituents are manufactured at constant rates relative to each other. If nutrient levels or other environmental conditions change, unbalanced growth results. During unbalanced growth, the rates of synthesis of cell components vary relative to one another until a new balanced state is reached. Unbalanced growth is readily observed in two types of experiments: shift-up, where a culture is transferred from a nutritionally poor medium to a richer one; and shift-down, where a culture is transferred from a rich medium to a poor one. In a shift-up experiment, there is a lag while the cells first construct new ribosome to enhance their capacity for protein synthesis. In a shift-down experiment, there is a lag in growth because cells need time to make the enzymes required for the biosynthesis of unavailable nutrients. Once the cells are able to grow again, balanced growth is resumed and the culture enters the exponential phase. These shift-up and shift-down experiments demonstrate that microbial growth is under precise, coordinated control and responds quickly to changes in environmental conditions(Maloe,2005).

When microbial growth is limited by the low concentration of a required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present. The rate of growth also increases with nutrient concentration but in a hyperbolic manner much like that seen with many enzymes. The shape of the curve seems to reflect the rate of nutrient uptake by microbial transport proteins. At sufficiently high nutrient levels, the transport systems are saturated, and the growth rate does not rise further with increasing nutrient concentration (Maloe, 2005).

2.2.3 Stationary phase

In a closed system such as a batch culture, population growth eventually ceases and the growth curve becomes horizontal. This stationary phase usually is attained by bacteria at a population level of around 10^9 cells per ml. Other microorganisms normally do not reach such high population densities. For instance, protist cultures often have maximum concentrations of about 10^6 cells per ml. Final population size depends on nutrient availability and other factors, as well as the type of microorganism being cultured. In the stationary phase, the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, or the population may simply cease to divide but remain metabolically active (Ingraham,2005).

Microbial populations enter the stationary phase for several reasons. One obvious factor is nutrient limitation; if an essential nutrient is severely depleted, population growth will slow. Aerobic organisms often are limited by O_2 availability. Oxygen is not very soluble and may be depleted so quickly that only the surface of a culture will have an O_2 concentration adequate for growth. The cells beneath the surface will not be able to grow unless the culture is shaken or aerated in another way. Population growth also may cease due to the accumulation of toxic waste products. This factor seems to limit the growth of many anaerobic cultures (cultures growing in the absence of O_2). For example, streptococci can produce so much lactic acid and other organic acids from sugar fermentation that their medium becomes acidic and growth is inhibited. Finally, some evidence exists that growth may cease when a critical population level is reached. Thus entrance into the stationary phase may result from several factors operating in concert (Neidhart,2005).

As we have seen, bacteria in a batch culture may enter stationary phase in response to starvation. This probably occurs often in nature because many environments have low nutrient levels. Procaryotes have evolved a number of strategies to survive starvation. Some bacteria respond with obvious morphological changes such as endospore formation, but many only decrease somewhat in overall size. This is often accompanied by protoplast shrinkage and nucleoid condensation. The more important changes during starvation are in gene expression and physiology. Starving bacteria frequently produce a variety of

starvation proteins**,** which make the cell much more resistant to damage. Some increase peptidoglycan crosslinking and cell wall strength. The Dps (D NA-binding *p* rotein from *s* tarved cells) protein protects DNA.

Proteins called chaperone proteins prevent protein denaturation and renature damaged proteins. Because of these and many other mechanisms, starved cells become harder to kill and more resistant to starvation, damaging temperature changes, oxidative and osmotic damage, and toxic chemicals such as chlorine. These changes are so effective that some bacteria can survive starvation for years. There is even evidence that *Salmonella enterica* serovar Typhimurium *(S. typhimurium)* and some other bacterial pathogens become more virulent when starved. Clearly, these considerations are of great practical importance in medical and industrial microbiology (Neidhart,2005).

2.2.5 Death phase

For many years, the decline in viable cells following the stationary phase was described simply as the "death phase." It was assumed that detrimental environmental changes such as nutrient deprivation and the buildup of toxic wastes caused irreparable harm and loss of viability. That is, even when bacterial cells were transferred to fresh medium, no cellular growth was observed. Because loss of viability was often not accompanied by a loss in total cell number, it was assumed that cells died but did not lyse.

2.3 Mechanism of bacterial adhesion and development

Biofilm growth is governed by a number of physical, chemical and biological processes. There are a number of mechanisms by which numbers of microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell–cell interactions and grow as a complex structure (Breyers & Ratner, 2004). Biofilm formation comprises a sequence of steps (Breyers & Ratner, 2004).

At present, processes governing biofilm formation that have been identified include (Fig. 1): 1. pre-conditioning of the adhesion surface either by macromolecules present in

the bulk liquid or intentionally coated on the surface; 2. Transport of planktonic cells from the bulk liquid to the surface; 3. Adsorption of cells at the surface; 4. Desorption of reversibly adsorbed cells; 5. Irreversible adsorption of bacterial cells at a surface; 6. Production of cell–cell signaling molecules; 7. Transport of substrates to and within the biofilm ; 8. Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. These processes are accompanied by cell growth, replication, and EPS production; 9. Biofilm removal by detachment or sloughing (Breyers & Ratner, 2004). The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables (Table 1). In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning film (Martial, & Degraeve, 2008). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and extracellular polymeric substances (EPS) production are important for biofilm formation and development (Parsek & Greenberg, 2005). An increase in flow velocity or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (Simo˜es, Sillankorva, *et al*., 2007).

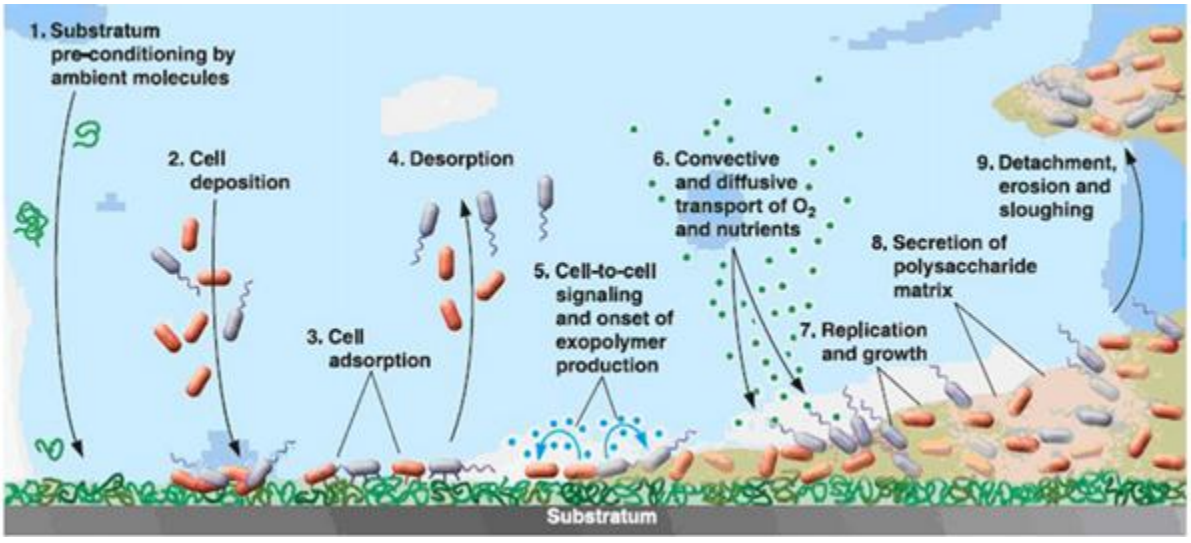

Figure 1: process of biofilm formation.

Table 2. 1: Variables important in cell attachment, biofilm formation and development (Donlan,2002)

Adhesion surface	Bulk fluid	Cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	Ph	Extracellular appendages
Surface chemistry	Temperature	Extracellular polymeric Substances
Charge	Cations	Signalling molecules
Conditioning film	Presence of antimicrobial product	
	Nutrient availability	

2.3.1 The conditioning layer

The conditioning layer is the foundation on which a biofilm grows, and can be composed of many particles, organic or inorganic. Anything that may be present within the bulk fluid can through gravitational force or movement of flow settle onto a substrate and become part of a conditioning layer. This layer modifies substrata facilitating accessibility to bacteria. Surface charge, potential and tensions can be altered favorably by the interactions between the conditioning layer and substrate. The substrate provides anchorage and nutrients augmenting growth of the bacterial community.

2.3.2 Cell–cell communication

The driving force in bacterial community development is the self-organization and cooperation among cells, rather than the classical 'competitive' natural selection of individual microorganisms (Parsek & Greenberg, 2005). This concept becomes particularly apparent when examining bacterial biofilm communities (Parsek & Greenberg, 2005). Cell– cell signalling has been demonstrated to play a role in cell attachment and detachment from biofilms (Daniels *et al*., 2004). Bacteria are considered to be far from solitary microorganisms, and in fact are colonial by nature and exploit elaborate systems of

intercellular interactions and communications to facilitate their adaptation to changing environments (Fuqua & Greenberg, 2002). The successful adaptation of bacteria to changing natural conditions is dependent on their ability to sense and respond to the external environment and modulate gene expression accordingly (Daniels *et al*., 2004).

Quorum sensing is based on the process of auto induction (Eberhard *et al*., 1981). The process of quorum sensing provides a mechanism for self-organization and regulation of microbial cells (Parsek & Greenberg, 2005). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, originally called an auto-inducer (AI) molecule, which accumulates in the surrounding environment during growth (Fuqua & Greenberg, 2002). Besides that, high cell densities result in high concentrations of signal, and induce expression of certain genes or physiological changes in neighboring cells (Parsek & Greenberg, 2005). A response to chemical signals in the process of cell communication is a concentration dependent process, where a critical threshold concentration of the signal molecule must be reached before a physiological response is elicited (Fuqua & Greenberg, 2002). Oligopeptides and N-acylhomoserine lactones (AHL) are major auto inducer (AI) molecules involved in intra-specific communication in Gram-positive and Gram-negative bacteria, respectively whereas boronated diester molecules (AI-2) are involved in inter-specific communication among both Gram-positive and Gram-negative bacteria (Parsek & Greenberg, 2005). Oligopeptides and N-acylhomoserine lactones (AHL) are the best characterized molecules (Ryan & Dow, 2008).

2.3.3 Population growth

As the stationary cells divide (binary division), daughter cells spread outward and upward from the attachment point to form clusters (Hall, 2002). Typically, such interactions and growth within the developing biofilm form into a mushroom-like structure. The mushroom structure is believed to allow the passage of nutrients to bacteria deep within a biofilm. After an initial lag phase, a rapid increase in population is observed, and cell growing exponential growth phase. This depends on the nature of the environment, both physically and chemically. The rapid growth occurs at the expense of the surrounding

nutrients from the bulk fluid and the substrate. At this stage the physical and chemical contribution to the initial attachment ends and the biological processes begin to dominate. Excretion of polysaccharide intercellular adhesion (PIA) polymers and the presence of divalent cations interact to form stronger bonding between cells (Dunme, 2002).

2.3.4 Final stages of biofilm development

The stationary phase of growth describes a phase where the rate of cell division equals the rate of cell death. At high cell concentration, a series of cell signaling mechanisms are employed by the biofilm, and this is collectively termed quorum sensing (Bassler, 1999). Quorum sensing describes as a process where a number of auto inducers (chemical and peptide signals in high concentrations, e.g. homoserine lactones) are used to stimulate genetic expression of both mechanical and enzymatic processors of alginates, which form a fundamental part of the extracellular matrix. The death phase sees the breakdown of the biofilm. Enzymes are produced by the community itself which breakdown polysaccharides holding the biofilm together, actively releasing surface bacteria for colonisation of fresh substrates.

2.4 Microbial Cell Surface Architecture

Since it is the microbial cell surface that largely determines the adhesion process it is necessary to describe a typical organization of the cell wall. Generally, a complete cell envelope possesses a number of functions (strength conferring, shape maintenance, molecular sieving, etc**.**) which can be provided by a single structural unit (Gram-positive bacteria) or by several layers with specialized functions (Gram-negative bacteria).

2.4.1 Gram-positive Bacteria

In Gram-positive bacteria**,** the stress-bearing component of the cell envelope that supports the internal turgor pressure of the cell is a thick, covalently cross-linked peptidoglycan-containing layer (Hancock, 1990). Other macromolecules such as polysaccharides, teichoic acids (secondary cell wall polymers), and proteins covalently linked to the peptidoglycan, penetrate its complex network. The relation between the amount of peptidoglycan (at least 40% by weight of the layer) and the total amount of anionic secondary polymers (remainder of the layer) with the outermost chains projecting into the surrounding fluid is generally maintained. So, the cell wall of Gram-positive bacteria is thought to be a covalently linked heteropolymeric structure overlaying and protecting the cytoplasmic membrane (Loeb, 1985). However, associated non-covalently with this structure are chemical components that represent extracellular products of the cell (glycocalyx). These are amphiphiles (lipoteichoic acids) that may retain an association with the cell membrane, wall-associated assemblies of glycoprotein forming regularly structured surface arrays (S-arrays) or capsules (‘slime layers ’) composed of an extracellular polysaccharide fibrous material.

2.4.2 Gram-negative Bacteria

While the cell wall of Gram-positive bacteria consist primarily of the relatively uniform single peptidoglycan-based layer, the cell wall of Gram-negative bacteria is multilayered and structurally and chemically more complex**.** Gram-negative bacteria possess a highly organized asymmetric outer membrane in which a bilayer of phospholipid (inner leaflet, 20-25%), lipopolysaccharide (oqter leaflet, 30%), and outer membrane protein (45-50%) constitute a permeability barrier with pores (ionic transmembrane channels) formed of aggregates of proteins (Hancock, 1991). So, the outer face of the outer membrane in the so-called smooth form (lipopolysaccharide consisting of a hydrophobic lipid component, a core polysaccharide, and 0-antigenicall y specific polysaccharide side chains) is hydrophilic. Interestingly, ‘rough’ mutants (lacking the core as well as the 0-polysaccharide portion of the lipopolysaccharide) are more hydrophobic and much more sensitive to hydrophobic molecules. Moreover, in Gram-negative bacteria, between the

outer cell membrane and the inner cytoplasmic membrane, there is a periplasm space filled with a macromolecular gel made up of a thin peptidoglycan layer in which periplasmic proteins and other molecules (lipoproteins) are distributed(Marshal,1985). Also, Gram-negative bacteria produce a wide variety of glycocalyces (glycoprotein S-arrays and polysaccharide capsules) closely associated with the cell surface.

2.5 Environmental factors influencing biofilm development

2.5.1 Effect of temperature

The optimum temperature for a microorganism is associated with an increase in nutrient intake resulting in a rapid formation of biofilm (Stepanovic, 2003). Nutrient metabolism is directly associated and dependent on the presence of enzymes. So it may be fair to say that the formation of a biofilm is dependent on the presence and reaction rates of enzymes, which control the development of many physiological and biochemical systems of bacteria. Temperature is correlated with the reaction rate of enzymes and the development of the cells. Optimum temperatures result in the healthy growth of the bacterial populations. Conversely, a temperature away from the optimum reduces bacterial growth. This is due to a reduction in enzyme to reaction rates. In addition, environmental temperature affects the physical properties of the compounds within and surrounding the cells. Fletcher (2001) reported the effect of temperature on attachment of stationary phase cells. Shown that a decrease in temperature reduced the adhesion of bacteria on the substrate. It is believed that the effect was due to a decrease in the bacterial surface polymer at lower temperatures as well as effects such as reduced surface area.

However, Herald and Zottola (1988) observed that the presence of bacterial surface appendages was dependent on temperature. At 35 ^{o}C cells were shown to have a single flagellum whilst at 21 ^{o}C they had two to three flagella and at 10 ^{o}C, cells exhibited on flagella. This may suggest that the initial interaction between the bacteria and substrate may increase with a lowering of temperature, increasing the likelihood of adhesion. Perhaps the more uniform properties of polysaccharides at lower temperatures increase the possibility of biofilm adhesion, because of many microbial polysaccharides undergo

transition from an ordered state at lower temperatures and in the presence of ions, to a disordered state at elevated temperature under low ionic environments.

2.6 Bacterial adhesion to surfaces

2.6.1 The influence of surface roughness.

Since the report in 1940 for Heukelekian (1940), has been known that the surface characteristics are an important factor for the bacterial adhesion and development. Until today this is central research area for the control of bacterial biofilm related disease. The adhesion of bacteria to a surface depends on a number of microbiological, physical, chemical, and material-related parameters, on surface topography has been widely produced as a parameter influencing bacterial adhesion (Flint, 1997). Contact with a solid surface induces the expression of a bacterial enzyme, which catalyzes the formation of exo-polysaccharides that promote colonization and protection. Thus, the modification of surfaces can be done to reduce attachment surfaces to limit the adhesion of microorganism e.g. electropolishing of stainless-steel. Several parameters or measures have been used to characterize the material surface based on two-dimensional characteristics such as the Ra (roughness average), Rt (is the maximum peak to valley height in the sample length), and Rz values (the average maximum profiler height) (Chiffre, 1990).

Amongst the most widely used is the surface roughness Ra value (which is the arithmetical mean deviation of the profile) and an Ra value of 0.8 µm or less has been recommended for dairies and, in general, for food contact surfaces. Although widely used, the Ra value will typically not characterize features of the surface such as soft or sharp topography or the presence of scratches or porosities During recent years, scanning electron microscopy (SEM) and atomic force microscopy (AFM) have been used to give a three-dimensional visualization of the surface topography including AFM determination of three-dimensional topographical parameters in the nanometer range (Stout, 1993).

2.6.2 Specialized attachment structures/surface properties of the cell

Cell surface hydrophobicity and the presence of extracellular filamentous appendages may influence the rate and the extent of microbial attachment. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing non-polar nature of one or both surfaces involved, for example the microbial cell and the adhesion surface (Donlan, 2002). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains. On the other hand bacteria and other microorganism have evolved many different ways to use the hydrophobic effect in order to adhere to surface (Doyle, 2002). Surface charges are inherently important for bacteria adhesion to the surface. In addition, bacteria may be affected by culture media, nutrients and age, the surface charge would also dependent on those parameters. Since it is the microbial cell surface that largely determines the adhesion process it is necessary to describe typical organization of the cell wall.

2.6.3 Electrostatic, Hydrophobic and Bridging Effects of Cell Surface Components

The reversible initial stage results from complex physicochemical interactions among the cell, the surface and the liquid phase (Kim and Frank, 1994). These interactions are caused by the surface charge (Hogt *et al*., 1985; Dickson and Koohamaraie, 1989), the hydrophobicity (Dahlback *et al*., 1981; Van Loosdrecht *et al*., 1987) and electron acceptor and electron donor (Van Oss, 1993) of interacting surfaces. The role of electron-donor/electron acceptor, i.e. Lewis acid-base proper- ties, in the interaction between two materials has been widely studied (Van Oss and Visser, 1992). Their importance in polar aqueous media has been underlined and reviewed by Van Oss (1993). Several studies (Boulangé-Petermann *et al*., 1993; Van Oss, 1993) have reported that the electron-donor/electron acceptor plays a crucial role in the microbial adhesion phenomenon. It should be noted that the energy of these interaction may be twice as much as that produced

by the Lifshitz-van der Waals interactions (LW) or electrostatic interactions (EL) usually described in the DLVO theory (Van Oss, 1996).

In 1996, Bellon-Fontaine *et al.* developed a new method-namely M.A.T.S (Microbial adhesion to solvents), to determine the electron donor/electron acceptor microbial cell properties. It was based upon the comparison between microbial cell affinity to a monopolar solvent and a polar solvent with the same LW surface tension component. This technique appears to be more useful than contact angle method (Van Oss *et al*., 1988), which requires specific and elaborate equipment. Microbial cell surface hydrophobicity is recognized as one of the determinant factors in microbial adhesion to surface (Van Loosdrecht *et al*., 1987). These properties are often evaluated by hydrophobic interaction chromatography, contact angle method, aqueous phase partitioning poly-ethyleneglycol/ dextran (PEG/DEX) and microbial adhesion to hydrocarbon (M.A.T.H). The latter technique is generally performed using p-xylene, hexadecane, octane and toluene. So, it can be a useful method to measure the cell surface hydrophobicity.

The cell surface physicochemical properties can be modified depending on surface cell structures (Ljunjh and Wadstrom, 1984; El Ghmari *et al*., 2002) or environmental factors such as temperature, medium composition, ionic strength and pH. Many workers have described the effects of these environmental parameters on hydrophobicity and charge (Beck *et al*., 1988; Herben *et al*., 1990; Van Der Mei *et al*., 1993; Latrache *et al*., 1994; Braindet *et al*., 1999a; Latrache *et al*., 2000). Literature data (Rouxhet and Mozes, 1990) reported that the hydrophobicity and charge were insufficient to explain the adhesion phenomenon. So the involvement of electron donor/electron acceptor properties could also be important in explaining this phenomenon (Van Oss *et al*., 1988). Despite the fact that the electron donor/electron acceptor properties play an important role in adhesion phenomenon, limited data concerning the effects of environmental parameters on these properties have been published (Braindet *et al*., 1999a; 1999b)